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Neurochemistry International 47 (2005) 573–579
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

Age-related changes in neutral sphingomyelin-specific phospholipase C activity in striatum, hippocampus, and frontal cortex: Implication for sensitivity to stress and inflammation

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Received 29 April 2004; received in revised form 17 May 2005; accepted 29 June 2005
Available online 2 September 2005

Abstract

Previous studies show the enrichment of mammalian brain with neutral sphingomyelin-specific phospholipase C (ceramide-phosphocholine phosphodiesterase, EC 3.1.4.12; N-Sase), a key enzyme of sphingolipid metabolism and sphingolipid-induced signaling.
Objective: The objective of this study was to evaluate the membrane-associated and cytosolic N-Sase activities in the brain regions associated with behavior (striatum, hippocampus, and frontal cortex).
Results: Results showed higher membrane-associated N-Sase activity as compared to the N-Sase activity in the cytosolic fractions of all the evaluated brain regions. In the hippocampus, the N-Sase activity was significantly higher than in the striatum and cortex. In addition, age-related changes in the hippocampal N-Sase activities were profoundly higher than in the respective fractions isolated from the striatum and cortex. Age-related decreases in the hippocampal and striatal cytosolic N-Sase activities were accompanied by increases in the membrane N-Sase activities in those brain regions. There was a significant increase in the cortical membrane-associated N-Sase activity with age; however, to a much lesser extend than in other brain regions. The increase in the hippocampal membrane-associated N-Sase activity was accompanied by a higher expression of the inflammatory marker, interleukin-1β (IL-1β), with age. One of the important findings of the present study is the region-specific expression of heat shock protein 70 (hsp70). Frontal cortex showed lower hsp70 expression in both young and old age groups as compared to the striatal and hippocampal hsp70 levels which can contribute to the recently reported higher cortical sensitivity to oxidative stress.
Conclusion: In conclusion (a) our results, for the first time to our knowledge, demonstrated the association between the N-Sase activity and the stress/inflammatory markers expression in the brain regions controlling behavior; (b) these findings suggest the role of N-Sase as a contributor to the increased stress and inflammatory sensitivity among the brain regions with age.

Keywords: Subcellular fractions; Heat shock protein 70; Cytokine interleukin-1β

1. Introduction

There is considerable evidence that aging is associated with a decline in the motor and cognitive behavior (Gallagher and Pelleymounter, 1988; Morris and McManus,

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0197-0186/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2005.06.011

1991; Rosenberg and Miller, 1992; Ingram et al., 1994; Colombo et al., 1997; Colombo and Gallagher, 2002; Albert, 2002). While the specific causes of behavioral decline are not known, we have been investigating in our studies the hypothesis that age-related alterations in the brain membrane lipid microenvironment (e.g., sphingolipids, etc.) may contribute to the increased brain vulnerability to stress and inflammation. This is an important consideration in the light of an emerging evidence implicating stress and inflammation as important contributors to brain aging and age-related behavioral deficit (for review see Joseph et al., 2001). Our previous data suggest that sphingolipid metabolites, such as ceramide and sphingosine, increased the production of reactive oxygen species in rat adrenal pheochromocytoma (PC12) cells, whereas sphingosine-1-phosphate decreased the levels of intracellular antioxidant, glutathione, and subsequently increased cellular vulnerability to oxidative stress (Denisova et al., 1999, 2001b). Numerous data implicated sphingolipid metabolites as important mediators of stress- and inflammatory-induced cellular signaling (Dressler et al., 1992; Obeid et al., 1993; Spiegel et al., 1996; Merrill et al., 1997; Ariga et al., 1998; Tomiuk et al., 1998; Dobrowsky, 2000; Marx, 2001; Brann et al., 2002). The interaction between sphingolipid-induced stress signaling and stress proteins [e.g., heat shock protein 70 (hsp70), cellular marker of neurotoxicity (Rajdev and Sharp, 2000)] has been recently demonstrated (Ahn et al., 1999). High expression of cellular stress proteins, including hsp70, has been suggested as a protective mechanism against stress-induced insults (Plumier et al., 1995; Marber et al., 1995; Radford et al., 1996; Plumier et al., 1997; Mattson, 2000a,b; Rajdev et al., 2000; Sato and Matsuki, 2002; for review see Calabrese et al., 2003).

It has been well documented that sphingolipid metabolite formation involves the activation of neutral sphingomyelin-specific phospholipase C (ceramide-phosphocholine phosphodiesterase, EC 3.1.4.12; N-Sase), a key enzyme in sphingolipid metabolism (Gatt, 1963, 1999; Hannun and Linardic, 1993; Spiegel and Milstien, 1995; Levade and Jaffrezou, 1999). Together with cytosolic Mg^{2+}-independent neutral sphingomyelinase, cytosolic Zn^{2+}-dependent acid sphingomyelinase, lysosomal acid sphingomyelinases, alkaline sphingomyelinase, and neutral sphingomyelin-specific phospholipase C, formed the sphingomyelinase family (Gatt, 1978; Liu et al., 1998b). These enzymes are distinguished by different pH optima, cellular topology, and cation dependence (Hannun et al., 2001). Recently, membrane-associated neutral sphingomyelinase has been implicated as an important effector enzyme in cytokine interleukin-1β (IL-1β)-induced signaling (Levade and Jaffrezou, 1999; Nalivaeva et al., 2000; Rybakina et al., 2001). However, there is no data to our knowledge regarding the association between the stress and inflammatory markers and the brain region-specific sphingomyelinase activity as a function of age.

Brain tissue is particularly enriched in N-Sase activity as compared to other tissues (Spence et al., 1979; Sperker and Spence, 1983; Tomiuk et al., 1998). Although rat brain N-Sase has been isolated, purified, characterized, and cloned (Liu et al., 1998a,b; Tomiuk et al., 1998; Liu and Hannun, 2000; Hofmann et al., 2000), the physiological role of this enzyme remains unknown. Earlier findings of high N-Sase activity in the striatum (Spence et al., 1979; Sperker and Spence, 1983), one of the brain regions controlling motor behavior and characterized with a high sensitivity to oxidative stress in aging (Shukitt-Hale et al., 1997; Joseph et al., 1998, 2001), suggested a possible enzyme involvement in the regulation of behavioral activity. Our recent data also demonstrated a highly significant negative correlation between the striatal N-Sase and Y-maze behavior in APP/PS-1 double transgenic mice (Denisova et al., 2001a). However, N-Sase activity in the brain regions associated with behavior and the effect of aging on subcellular enzyme activity was not systematically studied. Such data would provide a better insight into the role of sphingolipid metabolism and signaling in age-related vulnerability to stress and inflammation among the brain regions associated with behavior.

The present study was designed to test our working hypothesis that subcellular fraction-specific changes in N-Sase activity contribute to the vulnerability to stress and inflammation among the brain regions controlling behavior in aging. Therefore, N-Sase activities in membrane and cytosolic fractions isolated from the striatum, hippocampus, and frontal cortex were evaluated as a function of age. Stress and inflammatory brain region-specific sensitivity was evaluated through the expression of well-established stress (hsp70) and IL-1β markers as a function of age.

2. Experimental procedures

2.1. Materials

The reagents used were of the highest grade available. Non-fluorescent chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The Amplex Red fluorescent 96-well plate assay to measure neutral sphingomyelin-specific phospholipase C (ceramide-phosphocholine phosphodiesterase, EC 3.1.4.12; N-Sase) activity was obtained from Molecular Probes (Molecular Probes Inc., OR, USA; cat. #A-12220). Antibodies [i.e., heat shock protein (cat. #sc-1060) and interleukin-1β (cat. #sc-7884)] for the western immunoblotting procedure were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animals

Six-month-old (young) and twenty-two-month-old (old) male Fischer344 rats were obtained from a colony maintained by the National Institute on Aging. Rats were

housed individually in hanging, wire-mesh cages and maintained according to the regulations (see National Institute of Health, USPHS, Guide for the Care and Use of Laboratory Animals). These animals were used in compliance with all the applicable laws and regulations and principles expressed in the National Institute of Health, USPHS, Guide for the Care and Use of Laboratory Animals. This study was approved by the Animal Care and Use Committee of the Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University (Boston, MA).

2.3. Brain sample preparation

Rats were decapitated and brain tissues from three regions (striatum, hippocampus, frontal cortex) were dissected on ice. Three individual rats from each age group were assessed for brain region-specific N-Sase activity. Cytosolic and membrane fractions in our modification were isolated from individual brain regions (Kim et al., 1997; Denisova et al., 2000). Brain tissue was homogenized (50 mM Tris–HCl, pH 7.4, 2 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate) followed by low-speed centrifugation $1000 \times g$ for 15 min. The supernatant was centrifuged at $100{,}000 \times g$ for 1 h at 4 °C, and fractions were designated as cytosolic (supernatant) and membrane (pellet) fractions. Proteins were determined by using the Bio-Rad Protein Determination Kit (Bio-Rad Corp., Cambridge, MA). Each freshly isolated fraction was assessed for N-Sase activity.

In a parallel experiment, brain region-specific expression of stress (i.e., hsp70) and inflammatory (i.e., IL-1β) markers were assessed by western immunoblotting procedure. Striatal, hippocampal, and frontal cortical brain tissues from 10 individual young and 10 individual old rats were individually homogenized in a buffer: 50 mM Tris–HCl, pH 7.4, 2 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM orthovanadate, 0.5 mM DTT, 0.4 mM PMSF, 1 mg/mL leupeptin, 1 mg/mL pepstatin, and 1 mM sodium fluoride. Protein levels were determined, and aliquots of the homogenate with equal amounts of protein were assessed for hsp70 and IL-1β expression. The remaining homogenate was used for other assessments.

2.4. Neutral sphingomyelin-specific phospholipase C

Neutral sphingomyelin-specific phospholipase C (ceramide-phosphocholine phosphodiesterase, EC 3.1.4.12; N-Sase) activity was assessed in cytosolic and membrane fractions isolated from the striatum, hippocampus and frontal cortex (see Section 2.3) by using the Amplex Red fluorescent assay for 96-well plates according to the technical manufacturing procedure (Molecular Probes Inc., OR, USA) as described previously (Denisova et al., 2001b). Each reaction contained 50 μM Amplex Red reagent, 1 U/mL HRP, 0.1 U/mL choline oxidase, 4 U/mL of alkaline phosphatase, 0.25 mM sphingomyelin, and an aliquot (20 μg protein) of the brain sample. Sphingomyelinase from *Staphylococcus aureus* (0–40 mU/mL) was employed as a standard. Fluorescence was monitored on a Cyto Fluor Multi-Well Plate Reader (PerSeptive Biosystem, Framingham, MA) with $\lambda_{Ex/Em}$ at 530/590 nm.

2.5. Western immunobloting

The expression of stress (hsp70) and inflammatory (2B) markers was evaluated in aliquots of the striatal, hippocampal, and cortical homogenate by using standardized SDS-PAGE and immunoblotting procedures. Experimental samples for immunoblotting were adjusted for equal amounts of protein (80 μg protein) before their application on gels. The proteins recognized by specific antibodies (dilution 1:500) in immunoblots were then visualized by standard chemiluminescent's methods (Renaissance®, NEN-Life Science Products). Densitometric quantification of immunoblotted membranes was performed with Molecular Dynamics Densitometer (Amersham Biosciences, Sunnyvale, CA). The results were expressed as densitometric arbitrary units (DAU).

2.6. Statistical analyses

The results of biochemical measurements were analyzed by analysis of variance (ANOVA) and Fisher least-significant-difference tests. All statistical analyses were performed using Systat version 10.0 (SPSS Inc., Chicago, IL). All tests were two-sided. Results were considered statistically significant if the observed significance value was not greater than 0.05.

3. Results

3.1. Neutral sphingomyelin-specific phospholipase C

Fig. 1 represents N-Sase activity in young and old animals. N-Sase activity was evaluated in subcellular fractions isolated from the striatum, hippocampus, and frontal cortex. Membrane fractions were significantly enriched with N-Sase activity as compared to the cytosolic fractions across the brain regions. In addition, membrane-associated N-Sase activity was significantly higher with age in the evaluated brain regions: striatum ($P = 0.001$); hippocampus ($P = 0.018$); and frontal cortex ($P = 0.001$). Hippocampal membrane N-Sase activity was higher as compared to the striatal and cortical membrane N-Sase activity in both the age groups ($P < 0.001$) (Fig. 1A).

In contrast to the membrane-associated N-Sase activity, cytosolic enzyme activity decreased significantly with age in the striatum ($P = 0.023$) and the hippocampus ($P = 0.008$) (Fig. 1B) but not in the frontal cortex ($P = 0.426$). Striatal cytosolic N-Sase activity was significantly higher as

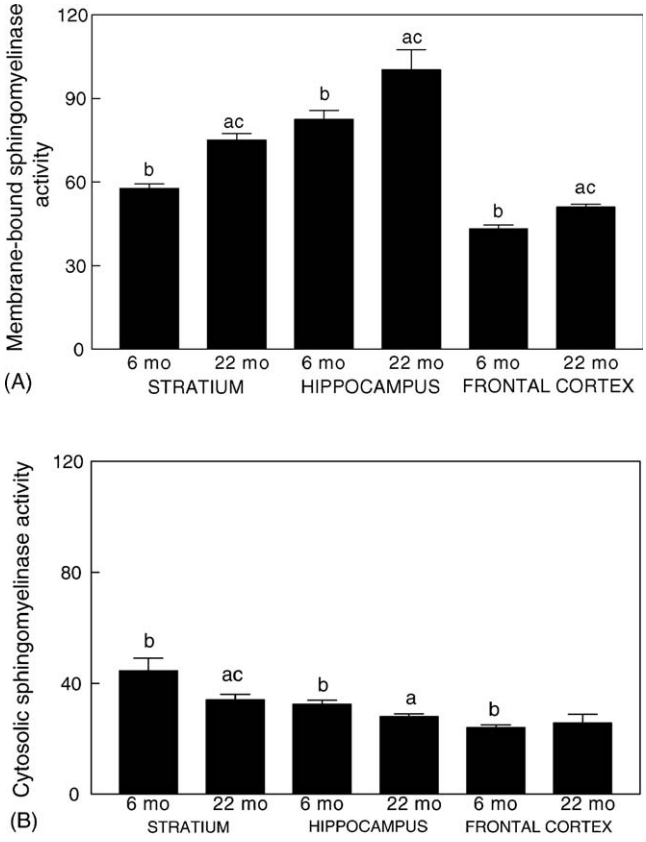

Fig. 1. Neutral phingomyelin-specific phospholipase C (ceramide-phosphocholine phosphodiesterase, EC 3.1.4.12; N-Sase) activity was assessed in membrane (A) and cytosolic (B) fractions isolated from the striatum, hippocampus, and frontal cortex, by using fluorescent method as described in Section 2. Fluoresce was monitored on a Cyto Fluor Multi-Well Plate Reader (PerSeptive Biosystem, Framingham, MA) with $\lambda_{Ex/Em}$ at 530/590 nm. Data are the means from three individual experiments ± S.E.M. (a) Age effect: the significant differences ($P < 0.05$) between the young and old animals within the same brain region (e.g., young striatum vs. old striatum, etc.). (b) Brain region-specific effect within young rats: the significant differences ($P < 0.05$) between the stiatum, hippocampus, and cortex among young group of animals. (c) Brain region-specific effect among old rats: the significant differences ($P < 0.05$) between the stiatum, hippocampus, and cortex within old group of animals.

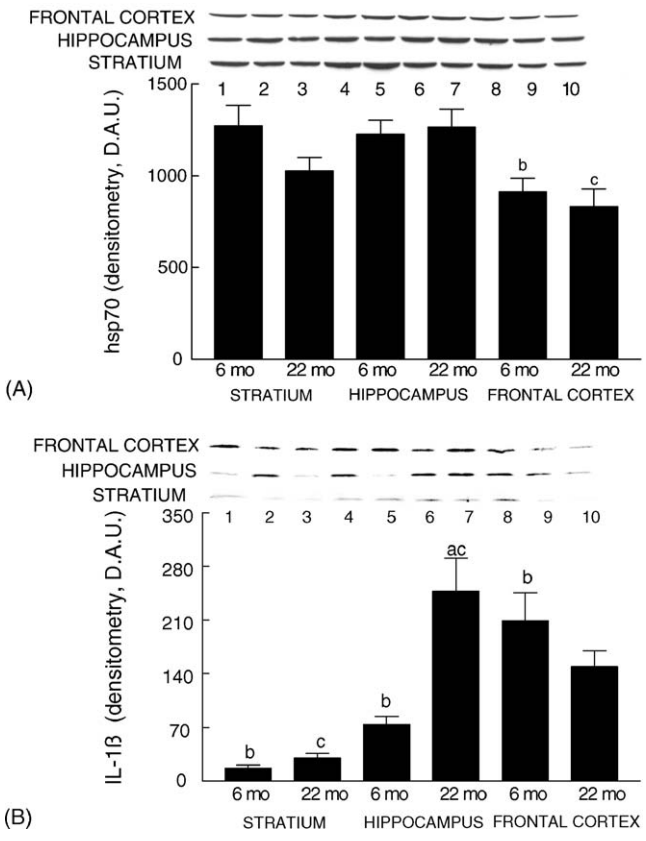

Fig. 2. The expression of stress (A) and inflammatory (B) markers was evaluated in the aliquots of the striatal, hippocampal, and cortical homogenate by using standardized SDS-PAGE and immunoblotting procedures. Experimental samples for immunoblotting were adjusted for equal amounts of protein (80 μg proteins) before their application on gels. Proteins recognized by specific antibodies (dilution 1:500) in immunoblots were then visualized by standard chemiluminescent's methods (Renaissance®, NEN-Life Science Products). Densitometric quantification of immunoblotted membranes was performed with Molecular Dynamics Densitometer (Amersham Biosciences, Sunnyvale, CA). The results were expressed as densitometric arbitrary units (DAU). The blots on the top of the figure represent the typical blot from five individual samples per each age group (i.e., samples #1–5—young rats, samples #6–10—old rats). Overall, data are the means from 10 individual experiments ± S.E.M. (a) Age effect: the significant differences ($P < 0.05$) between the young and old animals within the same brain region (e.g., young striatum vs. old striatum, etc.). (b) Brain region-specific effect within young rats: the significant differences ($P < 0.05$) between the stiatum, hippocampus, and cortex among young group of animals. (c) Brain region-specific effect among old rats: the significant differences ($P < 0.05$) between the stiatum, hippocampus, and cortex within old group of animals.

compared to the hippocampal and cortical N-Sase activity in both the age groups (young: $P < 0.001$; and old: $P < 0.01$).

3.2. The effect of aging on brain region-specific expression of stress and inflammatory markers

Fig. 2 represents the age-related distribution of stress (hsp70, Fig. 2A) and inflammatory (IL-1β, Fig. 2B) markers in the striatum, hippocampus, and frontal cortex. The blots on the top of each figure represent the typical blot from five individual samples per each age group (i.e., samples #1–5—young rats, samples #6–10—old rats). It appears that there were no significant changes in hsp70 expression as a function of age ($P = 0.339$) (Fig. 2A); however, there was a marginally lower significant expression of hsp70 in the striatum with age (young versus old; $P = 0.06$). Results showed a significant brain region-specific effect on the hsp70 expression ($P < 0.001$). Cortical hsp70 level was lower as compared to the striatal ($P = 0.007$) and hippocampal ($P = 0.017$) hsp70 levels in young rats. In old rats cortical hsp70 level was significantly lower as compared to hippocampal hsp70 levels ($P = 0.001$). There were no statistically significant differences between the striatal and hippocampal hsp70 expressions in either of the age group.

In young rats (Fig. 2B) cortical IL-1β expression was higher as compared to the striatal ($P = 0.001$) and hippocampal ($P = 0.001$) IL-1β expression. In old rats cortical and striatal IL-1β expression was lower as compared to the hippocampus ($P = 0.017$ and 0.001, respectively). In addition, a higher IL-1β expression as a function of age was observed only in the hippocampus ($P < 0.05$) (Fig. 2B).

4. Discussion

The present findings demonstrate a differential N-Sase activity in the brain regions associated with behavior. Consistent with the earlier studies (Spence et al., 1979; Sperker and Spence, 1983), our results demonstrated a high N-Sase activity in the striatum. The striatal N-Sase activity was almost two-fold higher than the N-Sase activity in the frontal cortex; however, it was lower as compared to the hippocampal enzyme activity. Previous research suggested that high N-Sase activity is an indicator of high sphingolipid metabolic activity (Spiegel and Milstien, 1995; Levade and Jaffrezou, 1999; Hannun et al., 2001). Therefore, our present data for the brain region N-Sase activity (hippocampal N-Sase activity > striatal N-Sase activity > cortical N-Sase activity) implicates the striatum and hippocampus as brain regions with high sphingolipid metabolism.

There is a strong association between the sphingolipids metabolism and the expression of stress (hsp70) (Ahn et al., 1999; Rajdev and Sharp, 2000) and inflammatory (IL-1β) markers (Lynch, 1998; Vereker et al., 2001). However, the exact mechanism of this association remains unknown. One of the important findings of the present study is the region-specific expression of hsp70. Frontal cortex showed lower hsp70 expression in both the young and old age groups as compared to the striatal and hippocampal hsp70 levels which can contribute to the recently reported higher cortical sensitivity to oxidative stress (Denisova et al., 2002). It appears that there is an inverse relation between hsp70 expression and the brain region vulnerability to oxidative stress. Lower hsp70 expression might be an indicator of high sensitivity to stress insults.

Consistent with the previous findings reported for hippocampal IL-1β expression (Lynch, 1998; Lynch and Lynch, 2002), our results demonstrated an increase in the hippocampal IL-1β expression with age. Importantly, our results, for the first time to our knowledge, demonstrated a brain region-specific association between the N-Sase activity and the stress/inflammatory markers. For example, in the hippocampus, high age-related membrane-associated N-Sase activity corresponded to a higher expression of inflammatory marker, IL-1β, whereas high striatal N-Sase activity was associated with a lower expression of the stress marker, hsp70 in old rats. The prevalent age-related increase in the striatal and the hippocampal N-Sase activity in the striatum and hippocampus may contribute to their high vulnerability to stress and inflammatory insults.

One important finding of the present study is the differential brain region-specific effect of aging on the N-Sase activity in cytosolic and membrane fractions. In the hippocampus brain regions particularly enriched with N-Sase activity, the effect of aging on the N-Sase activity was more profound as compared to those in the striatum and frontal cortex. Specifically, the hippocampal and striatal age-related decreases in cytosolic enzyme activity were accompanied by the increases in the membrane enzyme activities, whereas in the frontal cortex, only membrane-associated enzymes were up-regulated as a function of age. Our data support the previously reported up-regulation enzyme activity with age in the membrane fractions (Kim et al., 1997). In agreement with the previous findings in the rat liver and neuroblastoma cells (Hostetler and Yazaki, 1979; Spence et al., 1979, 1982; Das et al., 1984), the results of the present study demonstrated that in all the evaluated brain regions, the membrane fractions were significantly enriched with N-Sase activity as compared to the cytosolic fractions.

Although the determinant(s) contributing to the N-Sase activity across the brain regions remains unknown, possible brain-regional differences in glutathione levels (Denisova et al., 2000) and membrane lipid composition (Zhang et al., 1993, 1996; Wood et al., 1999; Joseph et al., 2001) can be accounted for the differential N-Sase activity. Our previous data demonstrated that the modification of the membrane lipid domains to the levels seen in some brain regions in aging induced N-Sase up-regulation accompanied by glutathione depletion (Denisova et al., 2001b).

Thus, our findings suggest that the differential effect of aging on the brain region-specific N-Sase activity may contribute to the sensitivity to insult among the brain regions associated with behavior.

Acknowledgments

This work was supported by ARS Cooperative Agreement #58-1950-9-001 to Tufts University and funded under CRIS #1950-51000-055. We thank Mrs. Beverly Dobson for her kind assistance in the manuscript preparation.

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